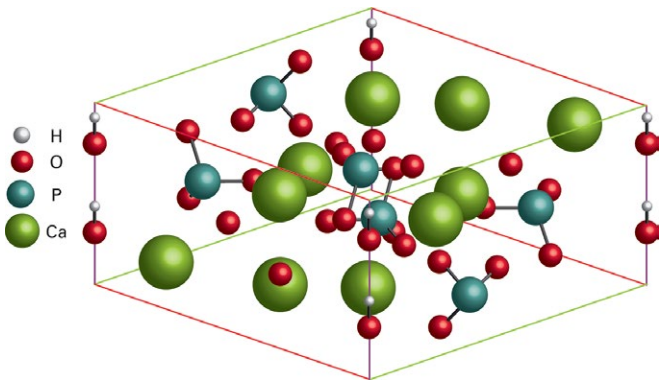
Ca^{++}Pure-HA
(Hydroxyapatite)

Ca++Pure-HA Resin

Ca++Pure-HA resin is a hydroxyapatite resin which has unique separation properties for biomolecules. This resin is specifically developed for the purification of antibodies and DNA; the separation of impurity antibody and its fragments from the native antibody (intact/monomer), and the isolation of single-stranded from double-stranded DNA.

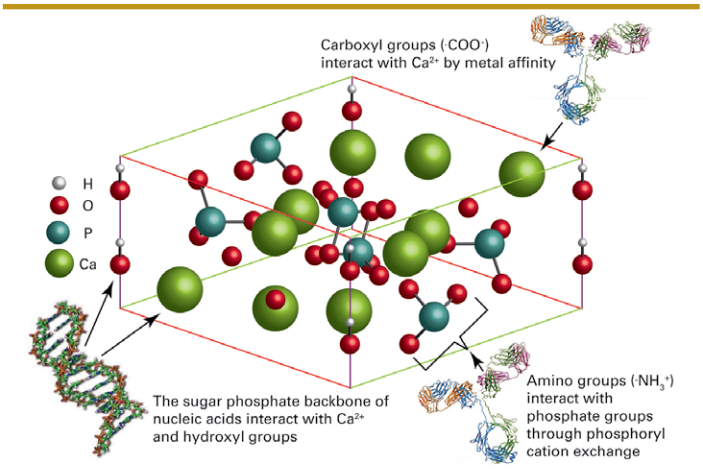
Ca++Pure-HA (hydroxyapatite: $Ca_{10}(PO_4)_6(OH)_2$) resin is a spherical, macroporous form of the hexagonal crystalline structure of hydroxyapatite (Figure 1). This resin has been sintered at high temperatures for increased mechanical and chemical stability, allowing it to withstand the rigors of industrial-scale applications. The robust nature of Ca++Pure-HA allows for it to be used reproducibly for many cycles at high flow rates and in large columns. Unlike other resins available from Tosoh Bioscience, the formation of the Ca++Pure-HA particle, both the ligand and the base bead, is created simultaneously from the same source of materials.

Figure 1: Ca++Pure-HA crystalline structure



Ca++Pure-HA resin has multiple mechanisms of interaction (Figure 2): calcium metal affinity interaction and phosphate group interaction.

Figure 2: Types of interactions with Ca++Pure-HA resin



The calcium metal affinity interactions occur between carboxyl (-OOC) groups on proteins and/or phosphate groups (e.g., sugar phosphate backbone of nucleic acids). These carboxyl and phosphate groups are repulsed by the negatively charged groups in the Hydroxyapatite structure. Elution of proteins that are bound by Ca^{2+} affinity (molecules that are composed mostly of acidic residues) will require increasing levels of phosphate in the mobile phase. Phosphate will out-compete proteins for the Ca^{2+} functional sites due to its strong affinity for calcium. Increasing concentrations of neutral salts, such as NaCl, will not typically have much effect on elution from the Hydroxyapatite resin when calcium metal affinity is the driving mechanism.

Cation exchange on Hydroxyapatite resin occurs when positively charged amino groups (^+H_3N) are ionically attracted to the negatively charged ***phosphate groups*** and are repulsed by the (Ca^{2+}) calcium groups. Elution of proteins bound by Hydroxyapatite resin through phosphoryl cation exchange (molecules that are composed mostly of basic residues), requires the addition of neutral salts, such as sodium chloride, to the mobile phase. Basic proteins may be eluted with either phosphates or neutral salts, depending on the selectivity of the resin for your target molecule and its impurities. Cationic interactions can also be disrupted by increasing mobile phase pH. Hence, the addition of salt or phosphate, or an increase in pH, can be used to weaken the interaction. In this way, Hydroxyapatite resin will behave in a similar fashion to a traditional cation exchange resin.

With its multiple mechanism of interaction and its unique particle formation, Ca++Pure-HA resin is used in the chromatographic separation of biomolecules and offers unparalleled selectivity and resolution for process scale operations. Its highly selective nature often separates proteins otherwise shown to be homogeneous by electrophoresis and other chromatographic techniques.

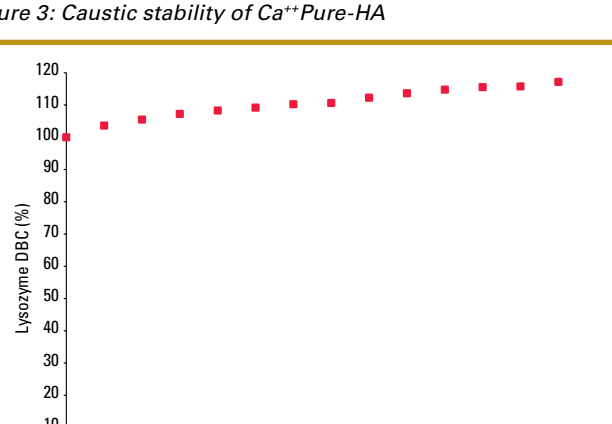
The key characteristics of Ca++Pure-HA resin are listed in Table 1.

Table 1: Properties of Ca++Pure-HA resin

Bead matrix	Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$
Particle size (mean)	39 µm
Dynamic binding capacity	> 30 g/L human IgG (2 min residence time) > 25 g/L lysozyme (2 min residence time)
Pressure rating	10 MPa (max)
Bead density	≥ 0.5 g/mL
Caustic stability	> 65 CIP cycles in 1.0 mol/L NaOH
Storage solution	Dry powder or 0.1 to 0.5 mol/L NaOH

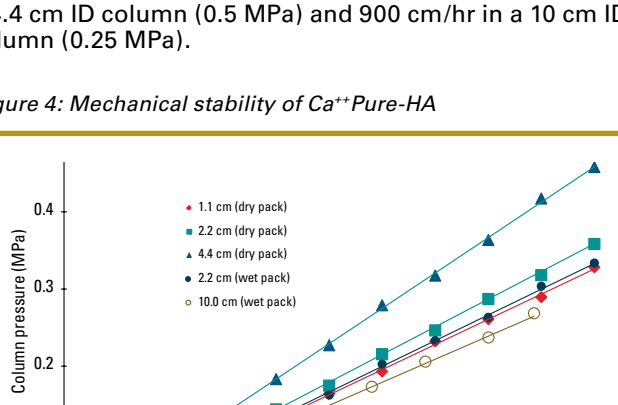
Ca⁺⁺Pure-HA is alkaline stable in 1.0 mol/L NaOH for greater than 65 CIP cycles with no appreciable loss of dynamic binding capacity (DBC). Figure 3 illustrates the caustic stability of Ca⁺⁺Pure-HA, with the DBC of lysozyme measured after every 5th CIP cycle with 1.0 mol/L NaOH.

Figure 3: Caustic stability of Ca⁺⁺Pure-HA



Ca⁺⁺Pure-HA is a rigid, crystalline support and can operate under very high flow rates and pressures when packed in a column. Ca⁺⁺Pure-HA was packed in columns of various ID to a height of 20 cm in a neutral pH mobile phase. Figure 4 demonstrates that Ca⁺⁺Pure-HA resin has excellent mechanical stability at linear velocities up to 1,000 cm/hr in a 4.4 cm ID column (0.5 MPa) and 900 cm/hr in a 10 cm ID column (0.25 MPa).

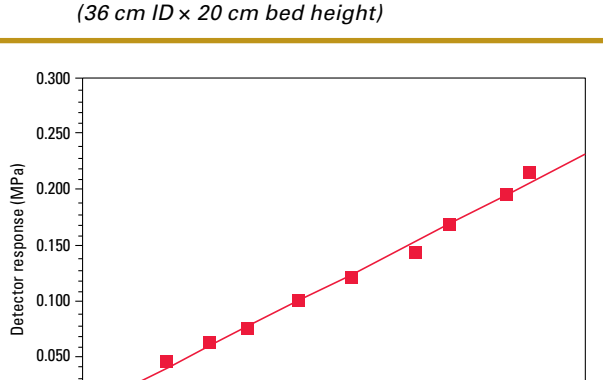
Figure 4: Mechanical stability of Ca⁺⁺Pure-HA



Resin:	**Ca⁺⁺Pure-HA**
Column size:	1.1 cm ID × 19.3 cm (dry pack) 2.2 cm ID × 20.3 cm (dry pack) 4.4 cm ID × 19.3 cm (dry pack) 2.2 cm ID × 20.6 cm (wet pack) 10.0 cm ID × 21.3 cm (wet pack)
Mobile phase:	5 mmol/L phosphate buffer, pH 7.2
Linear velocity:	as noted
Detection:	pressure (MPa)

To demonstrate the excellent pressure-flow in a process-scale column, Ca⁺⁺Pure-HA resin was packed in a 36 mm ID × 20 cm column. Data in Figure 5 shows that a flow rate of <0.3 MPa is achieved at a pressure drop at 600 cm/hr.

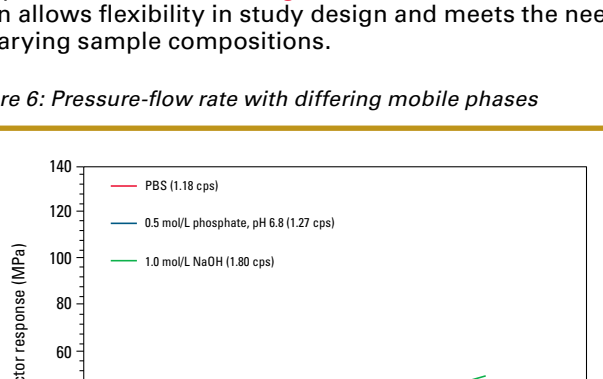
Figure 5: Pressure-flow rate curve on large process column (36 cm ID × 20 cm bed height)



Resin:	**Ca⁺⁺Pure-HA**
Column size:	36 mm ID x 20 cm
Mobile phase:	20 mmol/L phosphate buffer, 150 mmol/L chloride, pH 6.8
Linear velocity:	various
Detection:	pressure (MPa)

With solutions of differing viscosities, Ca⁺⁺Pure-HA exhibits low pressure, as shown in Figure 6. This characteristic of the resin allows flexibility in study design and meets the needs of varying sample compositions.

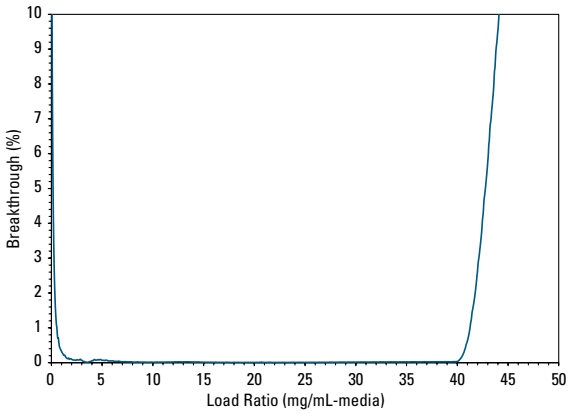
Figure 6: Pressure-flow rate with differing mobile phases



Resin:	**Ca⁺⁺Pure-HA**
Column size:	36 mm ID x 20 cm
Mobile phase:	as indicated in figure
Linear velocity:	various
Detection:	pressure (MPa)
Temperature:	23 °C

Ca++Pure-HA has a demonstrated a dynamic binding capacity (DBC), at 5% breakthrough, of greater than 40 g/L human IgG_1 at residence times as low as 4 minutes as shown in Figure 7.

Figure 7: Ca++Pure-HA dynamic binding capacity



Resin:	**Ca++Pure-HA , lot CPBL122716A**
Column size:	5 mm × 5 cm (1.0 mL)
Equilibration:	20 mmol/L MES, 5 mmol/L KPO_4, pH 6.5
Elution/Strip:	500 mmol/L KPO_4, pH 6.5
Sanitization:	1.0 mol/L KOH
Flow rate:	75 cm/hr (4 min residence time)
Detection:	UV @ 280 nm, Conductivity (mS/cm)
Temperature:	ambient
Sample:	IgG_1 @ 2.00 g/L
Instrument:	ÄKTA avant 25

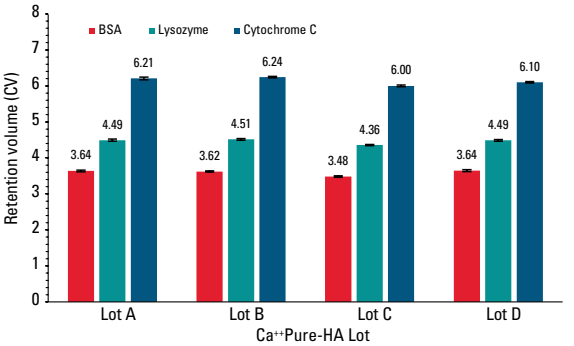
Table 2 shows the dynamic binding capacity (DBC) of IgG_1 at various residence times. Data shows that Ca++Pure-HA resin can achieve a DBC of greater than 30 g/L for human IgG_1 at 2 minutes residence time at 10% breakthrough.

Table 2: Dynamic binding capacity of Ca++Pure-HA for human IgG

	2 min	5 min
Human IgG	32.4 g/L	51.6 g/L

Ca++Pure-HA resin performs consistently from lot-to-lot, as shown in Figure 8. Separation of a protein mixture containing 1 g/L BSA, 0.2 g/L lysozyme, and 0.5 g/L cytochrome C was carried out in triplicate for four (4) individual lots of Ca++Pure-HA. Reproducible separation of the 3 standard proteins could be obtained under phosphate conditions.

Figure 8: Ca++Pure-HA lot-to-lot variability



Resin:	**Ca++Pure-HA**
Column size:	1.0 cm ID ×10 cm (7.9 mL)
Mobile phase	A: 1 mmol/L phosphate buffer, pH 6.8 B: 500 mmol/L phosphate buffer, pH 6.8
Gradient:	0–100% B (6 CV)
Linear velocity:	300 cm/hr (3.93 mL/min)
Residence time:	2.0 min
Detection:	UV @ 280 nm, conductivity
Temperature:	ambient
Injection vol.:	0.1 CV (0.8–0.9 mL)
Samples:	1 g/L BSA, 0.2 g/L lysozyme, 0.5 g/L cytochrome C in mobile phase A

Removal of mAb Aggregates

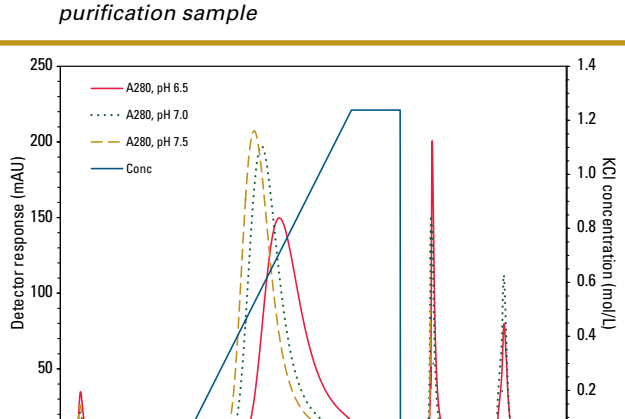
Downstream process chromatography scientists are constantly searching for better and more selective ways to remove aggregates and other process related impurities from a monoclonal antibody (mAb) monomer. Making use of chromatography resins with better selectivity, resolution and capacity is one approach to solving the problem of aggregate removal in monoclonal antibody production.

The following data demonstrates the capabilities of Ca^{++}Pure-HA media operated with potassium salts such as potassium phosphate and potassium chloride, to remove dimer and higher order aggregates from the monomer of a protein A purified IgG_1 monoclonal antibody.

To remove mAb aggregates from a post-protein A purified sample, Ca^{++}Pure-HA media was used in a polishing chromatography step. The below protocol was used.

Figure 9 shows a high resolution separation between the monomer peak and the aggregate peak across three different pH conditions using Ca^{++}Pure-HA media. The elution of the monomer peak at pH 6.5 was delayed and broader.

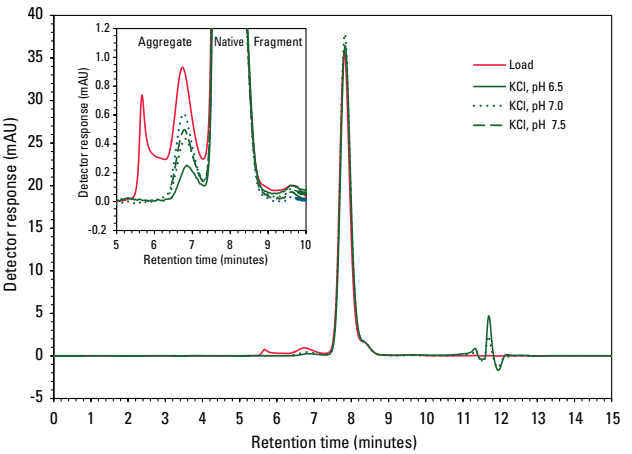
Figure 9: Removal of mAb aggregates from a post-protein A purification sample



Resin:	**Ca^{++}Pure-HA**
Column size:	5 mm × 5 cm (1.0 mL)
Mobile phase:	A: 50 mmol/L HEPES, 5 mmol/L KPO_4, pH as indicated B: mobile phase A + 2.0 mol/L potassium chloride, pH as indicated C: 500 mmol/L KPO_4, pH as indicated D: 1.0 mol/L KOH
Gradient:	69.4% B (chloride), 10 CV
Gradient:	delay, 5 CV
Flow rate:	300 cm/hr (1 min residence time)
Detection:	UV @ 280 nm, Conductivity (mS/cm), pH
Temperature:	ambient
Injection vol.:	5 µL
Sample:	2.0 mg/mL-media partially-purified mAb-01 (0.2 mL injection)
Instrument:	ÄKTA avant 25
Method:	Pre-equilibrate, mobile phase C, 3 CV
Equilibrate:	mobile phase A, 10 CV
	Load
	Wash, mobile phase A, 5 CV
	Elution, gradient as indicated, 25 CV
	Strip, mobile phase C, 5 CV
Sanitize:	mobile phase D, 5 CV

Size exclusion chromatography data analysis in Figure 10 show that after the sample passed through Ca^{++}Pure HA media under potassium phosphate buffer and potassium chloride operating conditions, mAb aggregates were reduced significantly. In fact, at pH 6.5 operating conditions, the aggregate amount was reduced from 6.6 to as low as 1.3%. Analytical HPLC peak integration data is shown in Table 3.

Figure 10: Aggregate analysis of pooled mAb monomer peaks eluted from different pH buffers using size exclusion chromatography



Column:	**TSKgel G3000SW$_{XL}$, 7.8 mm ID × 30 cm**
Mobile phase:	0.1 mol/L phosphate, 0.1 mol/L Na_2SO_4, 0.3% sodium azide, pH 6.7
Flow rate:	1.0 mL/min
Gradient:	isocratic
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection:	10 µg native
Instrument:	HPLC (400 bar pressure)

Table 3: Aggregate analysis data of pooled mAb monomer peak eluted from different pH conditions

Salt	pH	Peak molarity (mmol/L)	Recovery (% native)	Aggregate (%)	Fragment (%)
Load				**6.6**	**0.6**
KCl	6.5	814	72.9	1.3	0.5
	7.0	615	80.0	1.8	0.3
	7.5	509	81.0	2.2	0.3

Ordering Information

Ca^{++}Pure-HA resin:

Part #	Product description	Container size (g)
45045	Ca^{++}Pure-HA	50
45039	Ca^{++}Pure-HA	100
45040	Ca^{++}Pure-HA	250
45041	Ca^{++}Pure-HA	500
45042	Ca^{++}Pure-HA	1,000
45043	Ca^{++}Pure-HA	5,000